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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

12

Applicant's or agent's file reference PCT 99-45	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/19007	International filing date (day/month/year) 13 JULY 2000	Priority date (day/month/year) 13 JULY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): C12N 15/00 and US Cl.: 435/440		
Applicant MIDWEST RESEARCH INSTITUTE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and or drawings which have been amended and are the basis for this report and or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01 FEBRUARY 2001	26 OCTOBER 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/19007

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-9 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the claims:
pages 10 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the drawings:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the sequence listing part of the description:
pages 1-9 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4 [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5 [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No
PCT/US00/19007

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-8	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-8	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-8	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-8 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a mutated exoglucanase with reduced glycosylation due to the replacement of asparagine residues at positions 45, 270 or 384 in the amino acid sequence of a parent cellobiohydrolase.

---------- NEW CITATIONS ----------

NONE

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 3 and 4 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): Claims 3 and 4 refer back to a single claim plurals. Appropriate correction is required.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: PAUL J. WHITE
NATIONAL RENEWABLE ENERGY LABORATORY
1617 COLE BOULEVARD
GOLDEN, CO 80401

Received
OCT 2 0 2000
Legal Office

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference PCT/99-45	Date of Mailing *(day/month/year)* 19 OCT 2000 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US00/19007	International filing date *(day/month/year)* 13 JULY 2000
Applicant MIDWEST RESEARCH INSTITUTE	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46).

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO Jenny J Dexter

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PCT/99-45	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below
International application No. PCT/US00/19007	International filing date *(day/month/year)* 13 JULY 2000	(Earliest) Priority Date *(day/month/year)* 13 JULY 1999
Applicant MIDWEST RESEARCH INSTITUTE		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (See Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATION. SEARCH REPORT

In ational application No.
PCT/US00/19007

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/00
US CL :435/440

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/440, 435/209, 510 320

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA CAPLUS BIOSIS MEDLINE BIOTECHABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, E	US 6,114,296 A (SCHULEIN ET AL.) 05 September 2000 (05.09.00), see entire document.	1-8
Y	US 5,298,405 A (NEVALAINEN ET AL.) 29 March 1994 (29.03.94), see entire document.	1-8
Y	US 4,472,504 A (GALLO) 18 September 1984 (18.09.94), see entire document.	1-8
Y	EP 0,133,035 A2 (SHIN NENRYOYU KAIHATSU GIJUTSU KENKYU KUMIAI) 13 February 1985 (13.02.85) see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 SEPTEMBER 2000	Date of mailing of the international search report **19 OCT 2000**
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MANJUNATH RAO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/04284 A1

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(21) International Application Number: PCT/US00/19007

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(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/143,711 13 July 1999 (13.07.1999) US

(71) Applicant *(for all designated States except US)*: **MID-WEST RESEARCH INSTITUTE** [US/US]; 425 Volker Boulevard, Kansas City, MO 64110 (US).

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(74) Agent: **WHITE, Paul, J.**; National Renewable Energy Laboratory, 1617 Cole Boulevard, Golden, CO 80401 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/04284 A1

(54) Title: CELLOBIOHYDROLASE REDUCED GLYCOSYLATION VARIANTS: CBHIN45A; CBHIN270A; AND CBHIN384A

(57) Abstract: The invention provides a method for making an active exoglucanase in a eukaryotic heterologous host, the method comprising reducing glycosylation of the exoglucanase, wherein reducing comprises replacing an N-glycosylation site amino acid residue with non-glycosyl accepting amino acid residue. The invention further provides a cellobiohydrolase, comprising the reduced glycosylation variant cellobiose enzymes CBHIN45A; CBHIN270A; or CBHIN384A, or any combination thereof.

CELLOBIOHYDROLASE REDUCED GLYCOSYLATION VARIANTS: CBHIN45A; CBHIN270A; AND CBHIN384A.

Technical Field.

This invention relates to exoglucanases. More specifically, it relates to *Trichoderma reesei* cellobiohydrolase I reduced glycosylation variants which enable expression of the active enzyme in a heterologous host.

Background Art.

The surface chemistry of acid pretreated-biomass, used in ethanol production, is different from that found in plant tissues, naturally digested by fungal cellulase enzymes, in two important ways: (1) pretreatment heats the substrate past the phase-transition temperature of lignin; and (2) pretreated biomass contains less acetylated hemicellulose. Thus, it is believed, that the cellulose fibers of pretreated-biomass are coated with displaced and modified lignin. This alteration results in a non-specific binding of the protein with the biomass, which impedes enzymatic activity. Moreover, where the pretreated biomass is a hardwood-pulp it contains a weak net-negatively charged surface, which is not observed in native wood. Therefore, for the efficient production of ethanol from pretreated biomass it is desirable to enhance the catalytic activity of glycosyl hydrolases on acid hydrolyzed hardwoods.

Trichoderma reesei CBH I is a mesophilic cellulase enzyme, and comprises a major catalyst in the overall hydrolysis of cellulose. An artificial ternary cellulase system consisting of a 90:10:2 mixture of *T. reesei* CBH I, *A. cellulolyticus* EI, and *A. niger* β-D-glucosidase is capable of releasing as much reducing sugar from pretreated yellow poplar as the native *T. reesei* system after 120 h. This result is encouraging for the ultimate success of engineered cellulase systems, because this artificial enzyme system was tested at 50°C, a temperature far below that considered optimal for EI, in order to spare the more heat labile enzymes CBH I and β-D-glucosidase. In order to increase the efficiency of such artificial enzyme systems it is desirable to engineer new *T. reesei* CBH I variant enzymes capable of active expression in a heterologous host. The heterologous host *Aspergillus awamori*, could provide an excellent capacity for synthesis and secretion of *T. reesei* CBH I because of its ability to correctly fold and post-translationally modify proteins of eukaryotic origin. Moreover, *A. awamori* is believed to be an excellent test-bed for *Trichoderma* coding sequences and resolves some of the problems associated with direct site directed mutagenesis in *Trichoderma*.

In consideration of the foregoing, it is therefore desirable to provide variant cellulase

enzymes having enzymatic activity when expressed in an heterologous host.

Disclosure of Invention.

It is a general object of the present invention to provide variant cellulase enzymes having enzymatic activity when expressed in a heterologous host, such as a filamentous fungi or yeast.

Another object of the invention is to provide a variant exoglucanase characterized by a reduction in glycosylation when expressed in a heterologous host.

Another object of the invention is to provide an active cellobiohydrolase enzyme capable of expression in heterologous fungi or yeast.

It is yet another object of the invention to provide a method for reducing the glycosylation of a cellobiohydrolase enzyme for expression in a heterologous host.

The foregoing specific objects and advantages of the invention are illustrative of those which can be achieved by the present invention and are not intended to be exhaustive or limiting of the possible advantages which can be realized. Thus, those and other objects and advantages of the invention will be apparent from the description herein or can be learned from practicing the invention, both as embodied herein or as modified in view of any variations which may be apparent to those skilled in the art.

Briefly, the invention provides a method for making an active exoglucanase in a heterologous host, the method comprising reducing glycosylation of the exoglucanase, reducing glycosylation further comprising replacing an N-glycosylation site amino acid residue with a non-glycosyl accepting amino acid residue. The invention further provides a cellobiohydrolase, comprising the reduced glycosylation variant cellobiose enzymes CBHIN45A; CBHIN270A; or CBHIN384A, or any combination thereof.

Best Mode for Carrying out the Invention.

Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

A method for reducing the glycosylation of an expressed *Trichoderma reesei* CBHI protein by site-directed mutagenesis ("SDM") is disclosed. The method includes replacing an N-glycosylation site amino acid residue, such as asparagines 45, 270, and/or 384 of SEQ. ID NO: 4

(referenced herein as CBHIN45A, CBHIN270A and CBHIN384A, respectively), with a non-glycosyl accepting amino acid residue, such as is alanine. Various mutagenesis kits for SDM are available to those skilled in the art and the methods for SDM are well known. The description below discloses a procedure for making and using CBHI variants: CBHIN45A; CBHIN270A; and CBHIN384A. The examples below demonstrate the expression of active CBH I in the heterologous fungus *Aspergillus awamori*.

Industrial Applicability.

Site-Directed Mutagenesis of *Trichoderma reesei* CBH I for Reduced Glycosylation.

Aspergillus awamori was transformed with various versions of the *cbhl* gene from *Trichoderma reesei*. *The cbhl* genes included both cDNA and genomic (intron containing) versions. These were altered by site-directed mutagenesis for the specific purpose of reducing the glycosylation of the expressed CBH I protein through replacement of the N-glycosylation site amino acid residues (asparagine) with non-glycosyl accepting amino acid residues (alanine). The gene was propagated in an *E. coli* vector plasmid (pPFE2) under the control of the *Aspergillus awamori* glucoamylase promoter and signal sequence, and *trpC* terminator, and carrying resistance to ampicillin *(E. coli* selection) and Zeocin (Bleomycin) *Aspergillus* selection. One altered rCBH I variant, CBHIN270A, SEQ. ID. NO: 2, was isolated from cultures and determined to be consistent with native CBH I, SEQ. ID. NO: 4, with respect to kinetics on *p*NPL and was only slightly higher in molecular weight. Thus, construction of the triple reduced glycosylation mutant CBH 1, CBHIN270A (SEQ. ID. NO: 2) / CBHIN45A (SEQ. ID. NO: 1) / CBHIN384A (SEQ. ID. NO: 3), may provide a viable means of producing active CBH I in heterologous fungal or yeast which do not require the cellobiose/lactose induction cascade, known in *Trichoderma.* It is believed that reduced glycosylation CBH I mutants would also serve effectively in yeast-based high throughput screens, which are normally rendered unusable for fungal enzymes because of hyperglycosylation.

Example 1. Production of Active Recombinant CBH I (rCBH I) in *Aspergillus awamori*

Construction of Modified CBH I Coding Sequence.

The coding sequence for *T. reesei* CBH I (SEQ. ID. NO: 4) was successfully inserted and expressed in *Aspergillus awamori* using the fungal expression vector pPFE2 (and pPFE1). Vectors pPFE1 and pPFE2 are *E. coli-Aspergillus* shuttle vectors, and contain elements required for maintenance in both hosts. They encode ampicillin resistance for selection in *E. coli* and

Zeocin resistance for selection in *Aspergillus*. The foregoing provided for the site-directed mutagenesis in *E. coli*, followed by expression of the new mutant proteins in *A. awamori*. The CBH I gene is under the control of the *A. awamori* glucoamylase promoter and includes the glucoamylase secretion signal peptide. In order to have the signal peptide properly cleaved during secretion, the construction of this plasmid required the addition, by PCR, of a NotI site and XbaI site on the coding sequence of CBH 1. The NotI site addition resulted in a change of the most N-terminal amino acid on the protein from glutamine to glycine. This glycine was subsequently changed back to the native glutamine in the pPFE2/CBHI construct, using site-directed mutagenesis PCR. This new construct was used to transform *A. awamori* and to express rCBH I, as confirmed by western blot analysis of culture supernatant. The rCBH I expressed in *A. awamori* tends to be over glycosylated as evidenced by the higher molecular weight observed on western blot analysis. Over-glycosylation of CBH I by *A. awamori* was confirmed by digestion of the recombinant protein with endoglycosidases. Following endoglycosidase H and F digestion, the higher molecular weight form of the protein collapses to a molecular weight similar to native CBH 1.

The vector pPFE2/CBHI requires a relatively long PCR reaction (8.2 kb) to make site-specific changes using the Stratagene Quik Change protocol. The PCR reaction was optimized as follows using a GeneAmp PCR System 2400, Perkin Elmer Corporation. The reaction mixture contained 50 ng of template DNA, 125 ng each of the sense and antisense mutagenic primers, 5 µl of Stratagene 10x cloned *Pfu* buffer, 200µM of each: dNTP, 5 mM $MgCl_2$ (total final concentration Of $MgCl_2$ is 7 mM); and 2.5 U *Pfu* Turbo DNA polymerase. The PCR reaction was carried out for 30 cycles, each consisting of one minute denaturation at 96C°, 1 minute annealing at 69°C, and 20-minute extension at 75°C. There is an initial denaturation for 2 minutes at 96°C and a final extension for 10 minutes at 75°C, followed by a hold at 4°C. Agarose gel electrophoresis, ethidium bromide staining, and visualization under UV transillumination were used to confirm the presence of a PCR product.

PCR products were digested with restriction enzyme DpnI, to degrade un-mutagenized parental DNA, and transformed into *E. coli* (Stratagene Epicurian Coli Supercompetent XL-1 Cells). Amp^R colonies were picked from LB-Amp^{100} plates and mutations were confirmed by DNA sequencing. Depending on scale, plasmid DNA was purified using the Qiagen QiaPrep Spin Miniprep Kit or the Promega Wizard Plus MaxiPrep DNA Purification System.

Transformation of *Aspergillus awamori* with *Trichoderma reesei* CBH I coding sequence.

Aspergillus awamori spore stocks were stored at -70°C in 20% glycerol 10% lactose. After thawing, 200 µL of spores were inoculated into 50 mL CM broth in each of eight baffled 250 mL Erlenmeyer flask. The cultures were grown at 28°C, 225 rpm for 48 h. The mycelia were removed by filtration with sterile Miracloth, Calbiochem, San Diego, CA, and washed thoroughly with sterile KCM. Approximately 10 g of washed mycelia were transferred to 50 mL KCM + 250 mg Novozym234 in a 250 mL baffled Erlenmeyer flask. The digestion mixture was incubated at 30°C, 80 rpm for 16-18 h. Spheroplasts were filtered through Miracloth into 50 mL conical centrifuge tubes, pelleted at 2000xg for 15 min and re-suspended in 0.7M KCl by gentle tituration with a 25 mL pipette. This procedure was repeated once. After a third pelleting, the spheroplasts were resuspended in 10 mL KC, pelleted and resuspended in 0.5 mL KC using a wide-bore pipet tip. The washed spheroplasts were transformed by adding 12.5 µL PCM and 5 µL DNA (≈0.5 µg/µL) to 50 µL of spheroplast in sterile 1.5 mL Eppendorf tubes. After incubation on ice for 45 minutes, 0.5 mL of room temperature PCM was added to the transformation mixture and was mixed by tituration with a wide bore pipet tip. The mixture was incubated at room temperature for 45 minutes. One milliliter of KC was added and mixed. The mixture was allocated between four tubes of molten CM top agar at 55°C, which were each poured over a 15 mL CM170 plate. The plates were incubated at 28°C for 2-3 days. Subsurface colonies were partially picked with a sterile wide bore pipet tip, exposing the remaining part of the colony to air and promoting rapid sporulation. After sporulation, spores were streaked onto several successive CM100 plates. After a monoculture was established, heavily sporulated plates were flooded with sterile spore suspension medium (20% glycerol, 10% lactose), the spores were suspended and aliquots were frozen at -70°C. Protein production was confirmed and followed by western blot using anti-CBH I monoclonal antibodies and the Novex Western Breeze anti-mouse chromogenic detection kit (Novex, San Diego, CA). Extracting genomic DNA using the YeaStar Genomic DNA Kit (Zymo Research, Orange, CA) and carrying out PCR with *pfu*-turbo DNA polymerase (Stratagene, La Jolla, CA) and *cbhI* primers confirmed insertion of the gene.

Production of Recombinant Enzyme.

For enzyme production, spores were inoculated into 50 mL CM maltose medium, pH 5.0, and grown at 32°C, 225 rpm in 250 mL baffled flasks. The cultures were transferred to 1.0 L of CM maltose in 2,800 mL Fernbach flasks and grown under similar conditions. For large-scale enzyme production (>1 mg), these cultures were transferred to 10-L CM maltose in a New Brunswick BioFlo3000 chemostat (10-L working volume) maintained at: 20% DO; pH 4.5; 25°C;

and 300 rpm. The culture was harvested by filtration through Miracloth after 2-3 days of growth. For the 10-L fermentation broth, the filtrate was concentrated and dia-filtered into 20mM sodium acetate pH 5.0 by tangential flow ultrafiltration with an Amicon DC30 concentrator equipped with a single 10,000 MWCO hollow fiber cartridge (1.1mm I.D., 2.4 m^2 surface area). The retentate from the 10-L concentration or the filtrate from smaller cultures was clarified in an Amicon DC-2 concentrator by tangential flow filtration with two 0.1 μm hollow fiber cartridges (1.1 mm I.D., 0.03 m^2 surface area, Millipore, Bedford, MA). The permeate was further concentrated with an Amicon CH-2 concentrator equipped with three 10,000 MWCO hollow fiber cartridges (1.1 mm I.D., 0.03 m^2 surface area). The final concentrate was sterile filtered through a 0.45 μm filter and stored at 4°C until used.

The recombinant CBH I protein, SEQ. ID NO.: 4, was purified by passing the concentrated culture broth over two or three CBinD900 cartridge columns (Novagen, Madison, WI) connected in series using a Pharmacia FPLC loading at 1.0 mL/min. (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The cartridges were equilibrated in 20 mM Bis-Tris pH 6.5 prior to loading and washed with the same buffer after loading. The bound rCBH I was eluted with 100% ethylene glycol (3 mL/column) by hand, using a syringe. The eluted rCBH I was concentrated in an Amicon 10 mL stirred cell using a 25 mm PM10 membrane to <2.0 mL and loaded onto a Pharmacia SuperDex200 16/60 size-exclusion column. The mobile phase was: 20 mM sodium acetate; 100 mM sodium chloride; and 0.02% sodium azide, pH 5.0 running at 1.0 mL/min. The eluted protein was concentrated by stirred cell and stored at 4°C. Concentration was determined by A_{280} using the extinction coefficient and molecular weight calculated for individual proteins by the ProtParam tool on the ExPASy website (http://expasy.ch/tools/protparam.html. Below are the formulations for the various media described herein:

Clutterbuck's Salts (20X)

Na_2NO_3	120.0 g/L
KCl	10.4 g/L
$MgSO_4*7H_20$	10.4 g/L
KH_2PO_4	30.4 g/L

CM- Yeast Extract- 5g/L

Tryptone- 5g/L

Glucose- 10g/L

Clutterbuck's Salts-50mL

Add above to 900mL dH_20, pH to 7.5, bring to 1000mL

CM Agar- CM+ 20g/L Agar

CMK CM Agar+ 0.7M KCl

CM100- CM + 100 g/mL Zeocin (Invitrogen, Carlsbad, CA)

CM 1070- CM+ 170 g/mL Zeocin

KCl- 0.7M KCl

KC- 0.7M KCl + 50mM CaCI2

KCM- 0.7M KCl + l0mM MOPS, pH 5.8

PCM 40% PEG 8000, 50mM $CaCl_2$, 10mM MOPS pH 5.8

Example 2. Production of Reduced Glycosylation rCBH 1: Sites N270A; N45A; and N384A.

rCBHl/pPFE2 has been optimized using site-directed mutagenesis to achieve expression of native molecular weight CBH I in *A. awamori*. The QuickChange SDM kit (Stratagene, San Diego, Ca) was used to make point mutations, switch amino acids, and delete or insert amino acids in the native CBH1 gene sequence. The Quick Change SDM technique was performed using thermotolerant *Pfu* DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The procedure used the polymerase chain reaction (PCR) to modify the cloned CBH1 DNA. The basic procedure used a supercoiled double stranded DNA (dsDNA) vector, with an insert of interest, and two synthetic oligonucleotide primers containing a desired mutation. The oligonucleotide primers, each complimentary to opposite strands of the vector, extend during temperature cycling by means of the polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with a Dpn1 restriction enzyme. Dpn1 is specific for methylated and hemi-methylated DNA and thus digests the unmutated parental DNA template, selecting for the mutation-containing, newly-synthesized DNA. The nicked vector DNA, containing the desired mutations, was then transformed into *E. coli*. The small amount of template DNA required to perform this reaction, and the high fidelity of the *Pfu* DNA polymerase contribute to the high mutation efficiency and minimizes the potential for the introduction of random mutations. Three glycosylation-site amino acids on the protein surface were targeted for substitution of an alanine (A) residue in place of asparagine (N). Single site substitutions were successfully completed in the CBH I coding sequence at sites N45, N270, and N384, of SEQ. ID NO.: 4 by site-directed mutagenesis, and confirmed by DNA sequencing.

Table 1.

CONSTRUCT	HOST	MW	K_M	V_{MAX}
T. reesei	none	57.8 kDa	1.94	0.746
rCBH I wt cDNA#	*A. awamori*	63.3 kDa	2.14	0.668
rCBH I wt genomic	*A. awamori*	63.3 kDa	--	--
rCBH I N270A	*A. awamori*	61.7 kDa	2.25	0.489

As shown in **Table 1**, Western blot analysis of the supernatant, obtained from a single glycosylation-site mutant CBHIN270A (SEQ. ID NO.: 2) culture expressed in *A. awamori*, demonstrated that a decrease, to a lower molecular weight (61.7 kDa), in the amount of protein had occurred, as compared to the that in the wild type cDNA (63.3 kDa), and the wild type genomic DNA (63.3 kDa). These results demonstrate a reduction in the level of glycosylation in the reduced glycosylation mutant CBHIN270A, via expression in *A. awamori*. It is also shown, in the Table, that the CBHIN270A enzyme nearly retained its native enzymatic activity when assayed using the *p*NPL substrate. While not shown in the Table, variants CBHIN45A (SEQ. ID NO.: 1), and CBHI384A (SEQ. ID NO.: 3) have also demonstrated a reduction in amount of glycosylation and native activity when expressed from the heterologous host *A. awamori*.

Example 3. Production of Reduced Glycosylation rCBH 1: Double and Triple Mutants.

Double and triple combinations of this substitution have also been completed in the CBH I coding sequence (SEQ. ID NO.: 4) at sites N45, N270, and N384 by site-directed mutagenesis and confirmed by DNA sequencing. These double and triple-site constructs will also yield rCBH I enzymes with reduced glycosylation and, presumably, native activity.

Mutagenic Primers Used in Site-directed Mutagenesis PCR

Not1, Xbal insertion for vector construction

Mutagenic primers

C-terminal strand (Xbal): AGAGAGTCTAGACACGGAGCTTACAGGC

N-terminal strand (Notl):

AAAGAAGCGCGGCCGCGCCTGCACTCTCCAATCGG

Repair of NotI site to native sequence

Mutagenic primers

sense strand:GGCAAATGTGATTTCCAAGCGCCAGTCGGCCTGCACTCTCC

antisense strand:GGAGAGTGCAGGCCGACTGGCGCTTGGAAATCACATTTGCC

N45A glycosylation site mutation

Mutagenic primers

sense strand- GGACTCACGCTACGGCCAGCAGCACGAACTGC

antisense strand: GCAGTTCGTGCTGCTGGCCGTAGCGTGAGTCC

N270A glycosylation site mutation

Mutagenic primers

sense strand: CCCATACCGCCTGGGCGCCACCAGCTTCTACGGCCC

antisense strand: GGGCCGTAGAAGCTGGTGGCGCCCAGGCGGTATGGG

N384A glycosylation site mutation

Mutagenic primers

sense strand: GGACTCCACCTACCCGACAGCCGAGACCTCCTCCACACCCG

antisense strand:

CGGGTGTGGAGGAGGTCTCGGCTGTCGGGTAGGTGGAGTCC

The foregoing description is considered as illustrative only of the principles of the invention. Furthermore, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and process shown as described above. Accordingly, all suitable modifications and equivalents may be resorted to falling within the scope of the invention as defined by the claims which follow.

Claims

1. A method for making an active exoglucanase in a eukaryotic heterologous host, the method comprising reducing glycosylation of the exoglucanase, wherein reducing comprises replacing an N-glycosylation site amino acid residue with non-glycosyl accepting amino acid residue.

2. The method of claim 1, wherein the N-glycosylation site amino acid residues include asparagine 45, 270, or 384 of SEQ ID NO: 4 and the non-glycosyl accepting amino acid residue includes alanine.

3. The method of claims 1 wherein replacing comprises site-directed-mutagenesis.

4. The methods of claims 1 wherein the exoglucanase comprises a cellobiohydrolase.

5. An exoglucanase, comprising SEQ. ID. NO: 1.

6. An exoglucanase, comprising SEQ. ID. NO: 2.

7. An exoglucanase, comprising SEQ. ID. NO: 3.

8. An exoglucanase, comprising a combination of claims 5,6, or 7.

SEQUENCE LISTING

<110> Adney, William S.
Decker, Stephen R.
Lantz-McCarter, Suzanne
Baker, John O.
Vinzant, Todd B.
Nieves, Rafael A.
Himmel, Michael E.

<120> CELLOBIOHYDROLASE REDUCED GLYCOSYLATION VARIANTS: CBHIN45A; CBHIN270A; AND CBHIN384A

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19007

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/00
US CL :435/440
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/440, 435/209, 510/320

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA CAPLUS BIOSIS MEDLINE BIOTECHABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, E	US 6,114,296 A (SCHULEIN ET AL.) 05 September 2000 (05.09.00), see entire document.	1-8
Y	US 5,298,405 A (NEVALAINEN ET AL.) 29 March 1994 (29.03.94), see entire document.	1-8
Y	US 4,472,504 A (GALLO) 18 September 1984 (18.09.94), see entire document.	1-8
Y	EP 0,133,035 A2 (SHIN NENRYOYU KAIHATSU GIJUTSU KENKYU KUMIAI) 13 February 1985 (13.02.85) see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
12 SEPTEMBER 2000

Date of mailing of the international search report
19 OCT 2000

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